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MEMBRANE FRACTIONS FROM RAT HEPATOMA

II. IMMUNOCHEMICAL CHARACTERIZATION OF DETERGENT-SOLUBLE MEMBRANE ESTERASES, GLYCOSIDASES AND LEUCYL-β-NAPHTHYLAMIDASE

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SUMMARY

1. Two different microsomal fractions (rough tR, smooth tSa) and a plasma membrane fraction (tP) isolated from the 4-dimethylaminoazobenzene-induced rat hepatoma D23 were investigated with immunodiffusion methods combined with staining reactions for esterase, glycosidase and leucyl-β-naphthylamidase active antigens.

2. Two esterase active antigens, also present in liver membranes, were found in the hepatoma. One was precipitated from tSa and the other from tP extracts. The latter only hydrolyzed α-naphthyl acetate, while the former also attacked longer fatty acid ester substrates.

3. In contrast to liver plasma membranes the tP fraction contained all three glycosidases studied (β-glucuronidase, β-galactosidase and *N*-acetyl-β-glucosaminidase). These enzyme active antigens were also found in the tumour microsomes with varying distribution.

4. One leucyl-β-naphthylamidase active antigen was present in all fractions, while one additional antigen was only detected in liver microsomes.

INTRODUCTION

By means of immunological methods we previously investigated molecular identities of detergent-soluble enzymes in rat liver membranes. The main interest was focussed on a plasma membrane fraction prepared according to Emmelot *et al.*[1] and on three subfractions of microsomes[2]. The membranes were essentially derived from hepatocytes. With immunodiffusion methods, used in combination with histochemical staining reactions, it was possible to demonstrate the presence in the membranes of a large number of enzymatically active antigens[2–5]. Some of these antigens are unique for a particular type of membrane, while others are common to the membranes studied. It was further seen that some seemingly homogeneous enzyme reac-

Abbreviation: DMAB 4-dimethylaminoazobenzene.

tions are carried out by immunologically distinct molecules, all being able to catalyze the same reaction. The possibility of non-specific adsorption of the same enzymes to different carrier antigens could in most cases be excluded.

The work has now been extended to comprise membranes from a 4-dimethylaminoazobenzene (DMAB)-induced rat hepatoma, maintained by serial passages. The hepatoma was a gift from Professor Baldwin (Nottingham, England). Its maintenancin our laboratory and the preparation of tumour membrane fractions have been described in the first paper of this series[6].

The aim of this work was to compare the enzyme patterns of the hepatoma membranes with those of normal hepatocytes, using the tools and criteria previously established. Based on the findings presented in this and the following paper further work aims at an elucidation of (1) the microsomal electron transport system in relation to changes in metabolic requirements of the hepatoma cells and (2) the effects of changes in the enzymatic composition of the plasma membranes on tumour–host relationship.

MATERIALS AND METHODS

Membranes

Female Sprague–Dawley rats, starved over night, were decapitated. The livers were removed and used for preparation of membrane fractions. Microsomes were isolated and subfractionated into a ribosome-bearing, rough membrane fraction (R) and two different smooth membrane fractions (Sa and Sb)[2]. The R and Sa microsomes, used in this study, are known to be derived from the endoplasmic reticulum. The Sb fraction, which is of unknown origin, was not used in this study. Plasma membranes (P) were isolated as described by Emmelot *et al.*[1]. After isolation all membranes were sedimentated and washed twice in phosphate-buffered saline.

Hepatoma D23 nodules were harvested about 2–3 weeks after transplantation. Necrotic material was thoroughly rinsed away and nodules undergoing strong necrosis were not used. The isolation of hepatoma plasma membranes (tP) was carried out according to Emmelot and Bos[7]. Subfractionation of hepatoma microsomes into tR, tSa and tSb fractions and their electron microscopical and biochemical characterization was performed as described in the preceding paper[6]. The fractions were washed as described above.

The 10000 $\times$ *g* pellet from rat liver homogenate was used as the mitochondrial fraction. Lysosomes were isolated as described by Wattiaux *et al.*[8] with Triton WR 1339. The lysosomes were broken by repeated freezing and thawing. The membranes (L) were sedimented and washed in saline and the supernatant (Ls) was concentrated (with polyethyleneglycol).

Fetal liver homogenates in 0.25 M sucrose were prepared from fetuses at 15 days of gestation.

Antisera

Antisera against the membrane fractions were prepared as previously described[3,5]. They were called a-R, a-Sa, a-P and a-tR, a-tSa, a-tP, respectively. Antisera from 3 or 4 rabbits were pooled and exhaustively absorbed with lyophilized rat serum (2 times 3 mg/ml antiserum).

Extraction of membrane antigens

Homogenates and membrane suspensions were solubilized with 1% sodium deoxycholate and 0.5% Lubrol W (cetylpolyoxyethylene condensate, ICI, England). Approx. 70% of the membrane proteins were extracted by this procedure. The suspensions were kept in ice bath for at least 2 h prior to centrifugation at 105000 × *g* for 60 min. Protein content of the supernatants were determined according to Lowry *et al.*[9], with bovine serum albumin as standard. The extracts were adjusted to 8–10 mg protein per ml before immunological analysis.

Immunodiffusion

Immunoelectrophoresis in macroscale was performed with 0.05 M barbiturate buffer (pH 8.2) on 6 cm × 10 cm glass plates covered with 13 ml 1% agarose. A current of 4 mA per plate was applied and electrophoresis was run at 4 °C for 5 h. In microscale, the buffer was 0.1 M (pH 8.6) and the plates (2.6 cm × 7.6 cm) held 3.5 ml agarose. The current was adjusted to 1.5 mA per plate for 1 h. Double-diffusion experiments were carried out with 1% agarose in phosphate-buffered saline either according to Ouchterlony[10] or Piazzi[11]. Diffusion, washing and drying procedures have been described earlier[5].

Characterization of immune precipitates

Enzyme activities in the immune precipitates were visualized by histochemical stainings[2–5]. All results were confirmed by repeated analysis of several membrane preparations.

Non-specific esterase

Non-specific esterase (EC 3.1.1) activity was assayed according to Uriel[12] with either α-naphthyl acetate or α-naphthyl propionate as substrate. α-Naphthyl nonanoate was used to detect lipases[13], that is esterases attacking esters of long-chain fatty acids.

Glycosidases

Glycosidases were developed with an incubation medium described by Raunio[14]. Three different activities were assayed: β-glucuronidase (EC 3.2.1.31), β-galactosidase (EC 3.2.1.23) and *N*-acetyl-β-glucosaminidase (EC 3.2.1.30), using the substrates: 6-bromo-2-naphthyl-β-D-glucuronide, 6-bromo-2-naphthyl-β-D-galactoside and naphthalene-2(2′-acetamido-2′-deoxy-β-D-glucosidoxy)3-carboxy-2″,5″-dimethoxy-4″-chloroanilide*, respectively.

The staining reaction for L-leucyl-β-naphthylamidase (EC 3.4.11.1) activity was carried out according to Nachlas *et al.*[15] with L-leucyl-β-naphthylamide–HCl as substrate.

Quantitative enzyme determinations of α-naphthyl acetate-splitting esterases were made with the same kind of extracts as used in the immunodiffusion tests. Released α-naphthol was measured according to Nachlas and Seligman[16].

RESULTS

Our earlier studies on microsomes and plasma membranes were all carried out on outbred Sprague–Dawley rats, while the hepatoma D23 was propagated in

* The substrates were purchased from the Sigma Chem. Co., St. Louis, Mo., U.S.A.

an inbred Wistar strain. In order to establish whether the pattern of enzyme active antigens, as detectable by our methods, differed between the two strains, membrane fractions from both inbred Wistar rats and Sprague–Dawley rats were reacted with the antisera prepared against fractions from Sprague–Dawley rats. No qualitative differences in the antigens described in this and the following paper were found.

Previous work has established that the enzyme-active antigens studied herein, originate primarily from parenchymal cells of the liver[17,18]. They may be considered as genuine membrane antigens since they cannot be removed by repeated washings with saline. The question of non-specific adsorption of enzymes to different immune precipitates has been discussed previously[19].

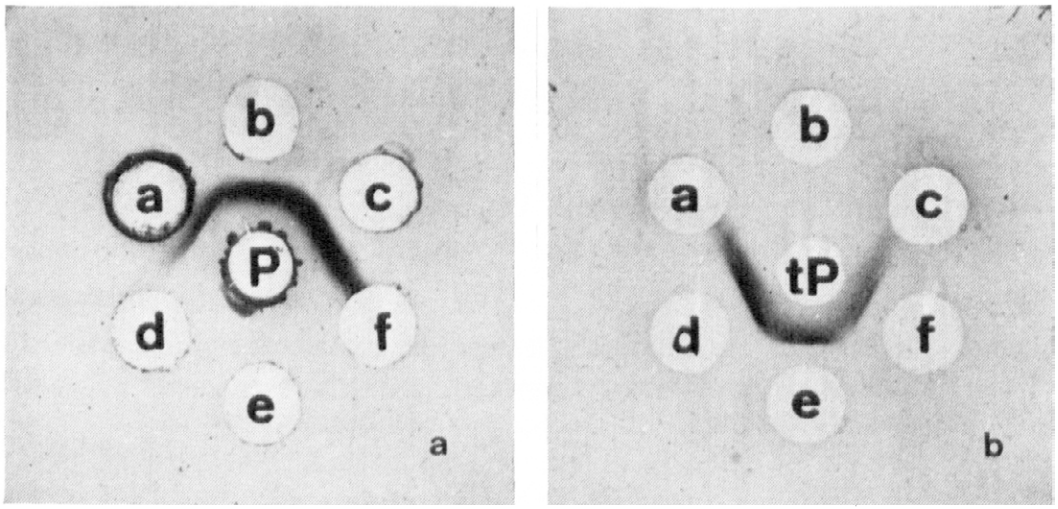

Fig. 1. Photographs of double-diffusion plates stained for non-specific esterase activity with α-naphthyl acetate as substrate. P and tP: detergent extracts of liver and hepatoma D23 plasma membranes respectively. a, b, c: antisera produced in 3 different rabbits against liver plasma membranes (a-P). d, e, f: antisera from 3 animals against D23 plasma membranes (a-tP). All antisera were absorbed with lyophilized rat serum.

Esterases

When plasma membranes from normal liver were reacted with their homologous antiserum (P/a-P) in double diffusion tests and stained for esterase activity with α-naphthyl acetate as substrate, one precipitate was stained (Fig. 1a). Occasionally it was possible to see that this line consisted of two precipitates (see Fig. 4) These precipitates did not develop in P/a-tP and tP/a-P reactions (Fig. 1a and b), the individual responses of the antibody-producing animals showing good correlation in this respect. The tP fraction contained, however, an immunologically different esterase, only precipitable with a-tP. This antigen could, however, also be precipitated by a-tP from liver mitochondrial and lysosomal fractions. The esterase hydrolysed only β-naphthyl acetate and not propionate or any of the longer fatty acids, indicating a different substrate specificity for this enzyme in contrast to the other esterases studied here. Interestingly, this esterase was not present in liver and hepatoma microsomes, as it could not be precipitated with a-tP from these fractions. Furthermore, a-tP only precipitated this esterase-active antigen from the Ls fraction of the liver lysosomes but not from the lysosomal membranes (L) (Fig. 2). Hepatoma lysosomes have not as yet been studied.

When tR and tSa were reacted with their homologous antisera, no esterase

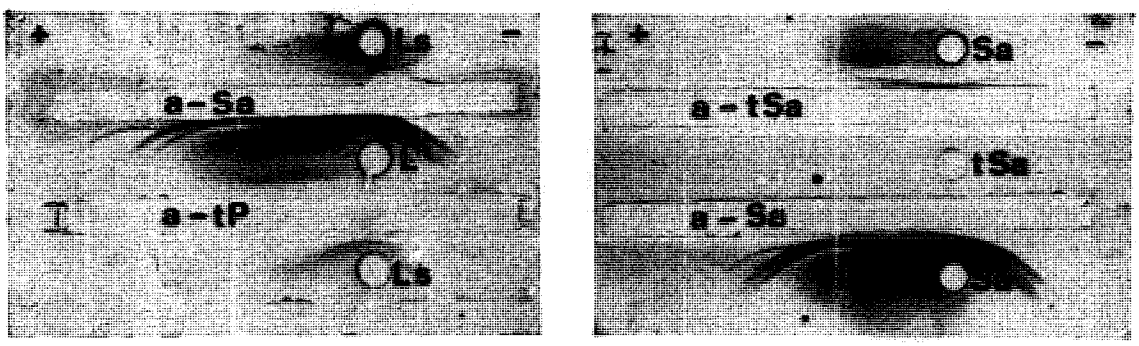

Fig. 2. Immunoelectrophoretic plate stained for esterase activity with α-naphthyl acetate. Membranes (L) and content (Ls) from liver lysosomes. a-tP and a-Sa: antisera to the corresponding fractions. The a-Sa precipitated all microsomal membrane esterases from the lysosomal membrane fraction. This could either depend on microsomal contamination of the lysosomal fraction or that the lysosomal membranes being related to the microsomes, also contain these esterases. The proper condition is at present unknown. The esterases precipitated by a-Sa and a-tP from Ls are immunologically different (shown in other experiments).

Fig. 3. Immunoelectrophoretic plate stained for esterase activity with α-naphthyl propionate as substrate. Sa and tSa: detergent extracts of smooth membranes from liver and hepatoma D23 microsomes, respectively. a-Sa and a-tSa: antisera against the fractions mentioned above.

active precipitates developed. One active precipitate was, however, obtained with Sa/a-tSa (see Fig. 3), indicating that one of the 8 esterase-active antigens of normal rat liver microsomes[20] was persisting in the tSa fraction in sufficiently large amounts to induce an antibody response. The concentration of this antigen in the extract was, however, too low for precipitation with either a-tSa or a-Sa. No esterase activity was precipitable from the tR membranes. Thus, most of the esterase-active antigens present in the liver R, Sa and plasma membranes have disappeared from the tumour or are present in too low amounts to be detected with the methods used in this study. The esterase present in tSa seems to be identical with the esterase active antigen appearing first in fetal liver. This is probably also identical with one of the adult plasma membrane esterases and is shared between rat liver and kidney (Fig. 4)[17,20].

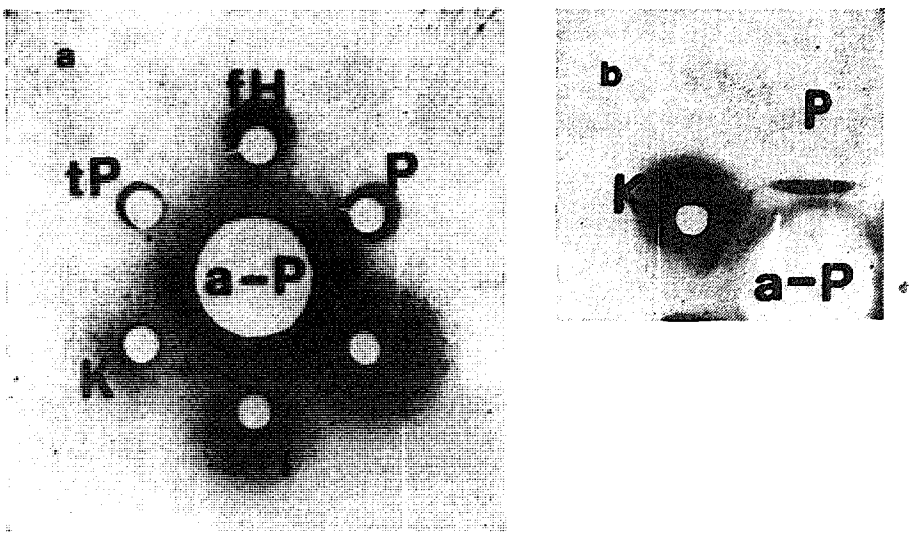

Fig. 4. Immunodiffusion plates, stained for esterase activity with α-naphthyl acetate as substrate. a-P: antiserum to liver plasma membranes. fH, aH and K: detergent extracts of fetal liver, adult liver and adult kidney homogenates, respectively. R: detergent extract of rough membranes from adult liver. For other abbreviations see text Fig. 1.

Furthermore this antigen was also found in the Ls fraction. It was, however, distinct from the esterase present in the tP fraction, as no immunological cross-reaction was seen between them. The microsomal esterase did not show the same substrate specificity.

All esterase active precipitates developed between Sa, R and P membranes and their homologous antisera stained for lipase according to Pascale *et al.*[13]. The esterase present in the tP-membrane fraction, however, did not give any staining with the relevant substrate, α-naphthyl nonanoate. This is in accordance with the finding that, in contrast to the other esterases, it only attacked α-naphthyl acetate but not α-naphthyl propionate.

Quantitative determinations of esterase activity in detergent extracts of the various fractions were carried out with α-naphthyl acetate as substrate. As could be expected, the tumour fractions showed considerably lower activity than the corresponding normal fractions. The largest difference was seen between tR and tSa and their normal counterparts (*e.g.* Sa 61.1, tSa 2.3 μmoles naphthol per mg per h). In contrast the P membranes were only about 4 times more active than the tP membranes (P 13.8, tP 3.0 μmoles naphthol per mg per h). The total homogenate of the tumour with and without detergent was not more active than the tumour fractions, suggesting that no rearrangement of esterases to other cell compartments had taken place during tumourogenesis. Thus, the decrease noted in the hepatoma seems to reflect a real loss of esterase activity.

Glycosidases

Antigens with various glycosidase activities like β-D-glucuronidase, β-D-galactosidase and *N*-acetyl-β-D-glucosaminidase were investigated both in R, Sa and P fractions of normal liver and in the corresponding fractions of the hepatoma.

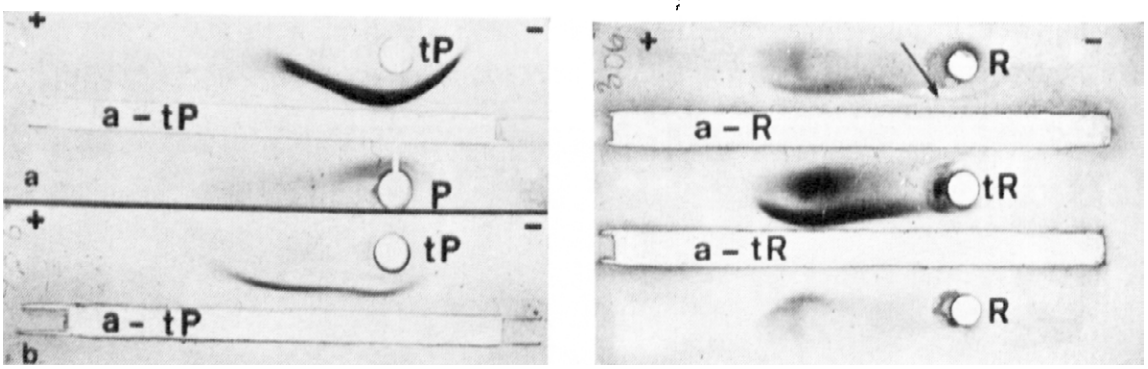

Fig. 5. Immunoelectrophoretic plates stained for (a) β-glucuronidase and (b) β-galactosidase activities. a-tP: antiserum to D23 plasma membranes. P and tP: detergent extracts of liver and D23 plasma membranes, respectively.

Fig. 6. Immunoelectrophoretic plate stained for leucyl-β-naphthylamidase activity. R and tR: detergent extracts of rough membranes from liver and D23, respectively. a-R and a-tR: antisera to the above-mentioned fractions. The arrow indicates leucyl-β-naphthylamidase active precipitate only found in liver microsomes.

One β-D-glucuronidase active antigen was precipitated from liver and tumour microsomes both with antisera against liver and tumour microsomes. An identity reaction was seen in double-diffusion tests between the various fractions. With a-R, a-Sa and a-tP but not with a-P, traces of the antigen were also detected in normal

plasma membranes. In contrast, hepatoma plasma membranes gave a strongly staining precipitate both with tumour and liver antisera, except with a-P (Fig. 5a).

β-Galactosidase and *N*-acetyl-β-D-glucosaminidase activities were also seen in one tP/a-tP precipitate (Fig. 5b) different from the β-glucuronidase active precipitate. It has so far been difficult to decide if both activities were in one precipitate or in two precipitates, laying close to each other. The two enzyme activities could not be precipitated from P membranes with any of the available antisera. This points to differences between these antigens and the β-glucuronidase active antigen. tSa and tR both gave a strongly β-galactosidase active precipitate with similar electrophoretic mobility as the tP/a-tP precipitate, when reacted with their antisera. In contrast Sa and R gave the same but much weaker reaction with their antisera. One *N*-acetyl-β-D-glucosaminidase active antigen was precipitated from tR and R with both a-tR and a-R, but no activity was found in Sa and tSa fractions. To investigate whether the glycosidases found in the tumour were of lysosomal type, liver lysosomal membranes and Ls fraction were reacted with a-tP and a-Sa, respectively. Surprisingly, no *N*-acetyl-β-D-glycosaminidase active precipitates could be detected in any combinations. A very weak galactosidase active precipitate appeared, however, with a-tP after soaking the plates for several hours in the staining mixtures. In contrast a strong glucuronidase active precipitate developed in all combinations within 30–60 min.

Leucyl-β-naphthylaminidase

This enzyme is known to be an intrinsic protein of liver and hepatoma plasma membranes[21,22]. By crosswise testing in immunoelectrophoresis, a highly active antigen was found in P as well as in tP. In contrast R and Sa membranes revealed two weakly stained precipitates when reacted with their homologous antisera (Fig. 6). One of these seemed to be identical with the enzyme-active precipitate from P membranes, while the other was immunologically different and only found in the microsomes (indicated by arrow in Fig. 6). This microsomal antigen was not detected in tumour microsomes, while the one shared with plasma membranes was present and showed an even stronger stainability in the tumour microsomes than in liver microsomes (tR/a-tR reaction in Fig. 6).

DISCUSSION

Loss of normal liver antigens from aminoazo-dye-induced hepatomas in rats and mice has been reported by a number of authors[23–27]. It has also been shown that the individual hepatomas differ in the number and types of antigens lost[27–29]. Baldwin[26], in his studies of a microsomal and a cell sap fraction from a DMAB-induced hepatoma (2nd or 3rd generation transplant), found that many of the normal liver antigens were deleted already at this stage. Baldwin and Barker[27], however, also detected "new" antigens present in both types of tumour fractions but not in normal liver or rat serum and recently Baldwin *et al.*[30] have also reported about a tumour-specific antigen present in the plasma membranes of hepatoma D23. Hirai *et al.*[31] previously also demonstrated new antigens in a soluble fraction from a transplanted rat hepatoma originally induced with DMAB, and Deckers[32] reported on abnormal microsomal antigens. The appearance of new tumour-specific transplanta-

tion (rejection) antigens on the cell surface of aminoazo-dye-induced hepatoma cells was demonstrated in syngenic combinations by transplantation methods[33] and also by immunofluorescence tests[27]. Concomitant with antigenic gain there is also a loss of normal liver antigens from the surface of the hepatoma cells, as defined by heteroantisera[34]. Moreover, these authors also presented some evidence indicating that different hepatoma lines, all characterized by their individual syngenically defined "tumour" antigens seem to have lost different "liver-specific" heteroantigens[34].

In most of these studies no further efforts were made to characterize the antigens involved. In this and the following paper, we have attempted to determine the nature of antigenic changes by combining histochemical staining reactions with immunodiffusion techniques. By comparing the antigens present in tumour membranes with their liver counterparts, further information on the biochemical and physiological implications of antigenic gains as well as antigenic losses in tumour cells has been obtained.

TABLE I

PRECIPITATES WITH ENZYME ACTIVITIES IN DIFFERENT FRACTIONS OF LIVER AND HEPATOMA D23

Enzyme	*Liver*		*Hepatoma*		*Remarks*
	Microsomes (R-Sa)	*Plasma membrane (P)*	*Microsomes (tR-tSa)*	*Plasma membrane (tP)*	
Esterases	8	2	1	1	tP antigen immunologically different from the tSa, P and Sa antigens but present in the content of liver lysosomes
Glycosidases					
Glucuronidase	1	—	1	1	all antigens immunologically identical
Galactosidase	1	—	1	1	strong activity in tP, tR and tSa; number of antigens unknown
N-Acetyl-β-glucosaminidase	1	—	1	1	
Leucyl-β-naphthylaminidase	2	1	1	1	one R and Sa antigen immunologically different from the other which is present in both P, tP, tR and tSa

A summary of the changes discussed in this paper is given in Table I. It will be apparent that the antigenic deletions (as well as gains) in hepatoma D23 comprised multiple components and entirely different enzyme systems. It should be said at this point that the cellular basis for these changes is at present unclear. It is thus possible that the hepatoma cells have lost components originally present in the hepatocytes from which they originated, or alternatively, the D23 cells were derived from cells lacking the antigen which is missing in the tumour.

Esterases

The hepatoma microsomes were depleted of all liver microsomal esterases

except one, which was shared with plasma membranes, lysosomes, fetal liver and kidney homogenates. The latter seemed to be the first esterase to appear during embryological development. Our results suggest that this esterase active antigen takes part in some fundamental reactions, the nature of which is unknown, but which are common to adult liver, fetal liver, kidney and tumour cells. The other esterases in liver microsomes seem to be involved in liver-specific reactions, not performed by any other cell type but the mature hepatocyte[2,20].

The hepatoma plasma membrane fraction on the other hand contained another esterase, which was found in the content of liver lysosomes but not in lysosomal membranes or microsomes. This esterase was substrate specific in that it could only hydrolyze α-naphthyl acetate and not any of the longer fatty acid esters tested. Emmelot and Bos[35] have also reported on differences between membrane esterases from rat hepatoma 484 and normal liver. Using α-naphthyl caprylate and α-naphthyl laurate as substrates they found that the hepatocellular membranes were able to hydrolyze caprylate at a 30–40 times higher rate than laurate, while the corresponding rate difference was only 6–7-fold in the tumour membranes.

The different distribution in the tumour fractions of the two esterases, both present in liver lysosomes, is probably physiologically significant and does not merely reflect a lysosomal contamination, as in that case both esterases should have been present in all fractions concerned.

Glycosidases

Some of the neoplastic transformations involving surface structures have been shown to depend on modifications in the arrangement of glycolipids, glycoproteins, or carbohydrates[36–38]. This may reflect a change in the enzymes involved in carbohydrate metabolism. The reports by Cumar *et al.*[39] and Grimes[40] on decreases in tumours of glycolipid: *N*-acetylgalactosaminyl transferase and glycoprotein-glycolipid:*N*-acetylneuraminyl transferase, respectively, favour this. Furthermore, Bosmann[41] found that several glycosidases were increased in transformed cells. Our results also indicate an increased glycosidase activity in the hepatoma. In normal liver, glycosidases are mainly known as lysosomal enzymes; high activities of *N*-acetyl-β-glucosaminidase and β-galactosidase have been reported[42]. Glycosidases are, however, also present in microsomes[43]. It was therefore of interest to note that all three glycosidases tested in this study were precipitated from the hepatoma plasma membrane fraction, while hardly any could be detected in liver plasma membranes. The microsomal fractions contained all three with somewhat varying distributions. The appearance of glycosidase active antigens in the hepatoma D23 plasma membrane fraction may either mean that these enzymes are plasma membrane associated or that the tumour fraction was contaminated with lysosomal membranes to a greater extent than the normal liver plasma membranes. Considering the distribution of esterases and the poor cross-reactivity of lysosomes with antisera against liver microsomes and hepatoma plasma membranes, lysosomal contamination as such of the hepatoma plasma membrane fraction is less likely. This weak cross-reactivity, despite high lysosomal glycosidase activities, could either mean that the enzymes present in liver microsomes and hepatoma membranes are different from the lysosomal enzymes or that there exist several species of glycosidases, some of which are predominating in lysosomes, while others are present in the other liver and tumour membranes.

Leucyl-β-naphthylamidase

This activity was found in one precipitate both in microsomes and plasma membranes from hepatoma and liver. Histochemical studies of liver tissue have demonstrated that the activity is located adjacent to the bile canaliculi[44,45]. Most of the plasma membrane activity was found in the globular knobs[21,22]. In hepatomas, which are less differentiated and lack developed bile canaliculi, the leucyl-β-naphthylamidase seems to be distributed differently[21].

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